

N-Acetylglucosamine Inhibits T-helper 1 (Th1)/T-helper 17 (Th17) Cell Responses and Treats Experimental Autoimmune Encephalomyelitis^{*[5]}

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Background: Multiple sclerosis (MS) has been linked to genetic and environmental dysregulation of Golgi *N*-glycosylation.

Results: Oral treatment of mice with the sugar *N*-acetylglucosamine (GlcNAc) enhances *N*-glycosylation, suppressing inflammatory T cell responses and an MS-like disease when initiated after disease onset.

Conclusion: Disease progression is suppressed by GlcNAc.

Significance: GlcNAc may provide the first MS therapy that directly targets an underlying mechanism causal of disease.

Current treatments and emerging oral therapies for multiple sclerosis (MS) are limited by effectiveness, cost, and/or toxicity. Genetic and environmental factors that alter the branching of Asn (*N*)-linked glycans result in T cell hyperactivity, promote spontaneous inflammatory demyelination and neurodegeneration in mice, and converge to regulate the risk of MS. The sugar *N*-acetylglucosamine (GlcNAc) enhances *N*-glycan branching and inhibits T cell activity and adoptive transfer experimental autoimmune encephalomyelitis (EAE). Here, we report that oral GlcNAc inhibits T-helper 1 (Th1) and T-helper 17 (Th17) responses and attenuates the clinical severity of myelin oligodendrocyte glycoprotein (MOG)-induced EAE when administered after disease onset. Oral GlcNAc increased expression of branched *N*-glycans in T cells *in vivo* as shown by high pH anion exchange chromatography, MALDI-TOF mass spectroscopy and FACS analysis with the plant lectin L-phytohemagglutinin. Initiating oral GlcNAc treatment on the second day of clinical disease inhibited MOG-induced EAE as well as secretion of interferon- γ , tumor necrosis factor- α , interleukin-17, and interleukin-22. In the more severe 2D2 T cell receptor transgenic EAE model, oral GlcNAc initiated after disease onset also inhibits clinical disease, except for those with rapid lethal progression. These data suggest that oral GlcNAc may provide an inexpensive and nontoxic oral therapeutic agent for MS that directly targets an underlying molecular mechanism causal of disease.

chronic neurological symptoms. Current treatment strategies for MS are predominated by injectable therapies targeting inflammatory demyelination, including first-line therapies glatiramer acetate (Copaxone; Teva) and β -interferons (Betaseron, Avonex, and Rebif). These agents have modest efficacy, high cost, and side effects such as injection site reactions, which can affect tolerability and compliance. Fingolimod (Gilenya; Novartis) is the first orally active treatment for MS, but serious adverse effects have been observed (1, 2). Other oral agents are also in development, including cladribine, which has shown efficacy as well as significant toxicity (3). Importantly, these new oral agents broadly and nonspecifically suppress the immune system, a questionable therapeutic approach given our increasing understanding of the pathogenic mechanisms promoting disease. The limitations of current medications highlight the need for safer, more convenient, and less costly treatment strategies. A simple oral therapy that promotes self-tolerance by targeting an underlying disease mechanism, rather than broad and nonspecific immunosuppression, remains a major unmet need for the management of MS.

As a complex trait disease, multiple genetic and environmental factors combine to determine MS susceptibility (4–6). Significant progress in understanding these mechanisms has been made in recent years. Genome-wide association studies have identified a number of genes associated with MS susceptibility, including variants of IL-2 receptor- α (*IL2RA*, rs2104286) and IL-7 receptor- α (*IL7RA*, rs6897932) (7–9). *MGAT5*, a gene encoding an enzyme in the Asn (*N*)-linked protein glycosylation pathway, was recently identified in a genome-wide association study for variants regulating MS severity (10). We recently demonstrated that the *IL2RA* and *IL7RA* MS risk variants alter *N*-glycan branching by blocking IL-2 and IL-7 signaling-mediated changes in *MGAT1*, a Golgi gene upstream of *MGAT5* (11). Moreover, an MS-associated variant of *MGAT1*

Multiple sclerosis (MS)² is characterized by inflammatory demyelination and neurodegeneration producing acute and

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² The abbreviations used are: MS, multiple sclerosis; CTLA-4, cytotoxic T lymphocyte antigen-4; EAE, experimental autoimmune encephalomyelitis;

GlcN, glucosamine; HPAEC, high pH anion exchange chromatography; MOG, myelin oligodendrocyte glycoprotein; PHA, phytohemagglutinin; TCR, T cell receptor; Th, T-helper.

interacts with multiple MS modulators to control *N*-glycan branching and MS risk, including the *IL2RA* and *IL7RA* risk variants and vitamin D₃ (11). Vitamin D₃ inversely associates with MS, regulates *MGAT1*, and inhibits experimental autoimmune encephalomyelitis (EAE) by promoting *N*-glycan branching (11). *Mgat5* deficiency in 129/Sv mice results in enhanced susceptibility to EAE and spontaneous kidney autoimmunity (12). Several mouse strains highly susceptible to EAE (PL/J, SJL, and NOD) display *N*-glycan branching deficiency in T cells compared with resistant strains (129/Sv, BALB/c, and B10.S) (13). The PL/J strain displays the lowest levels, with a small minority developing spontaneous late onset motor weakness characterized by inflammatory demyelination, neuronophagia, and axonal damage in demyelinated lesions and otherwise normal appearing white matter; phenotypes markedly enhanced by *Mgat5*^{+/-} and *Mgat5*^{-/-} genotypes in a gene dose-dependent manner. *N*-Glycan branching in neurons directly controls neuronal survival independent of inflammation, as neuron-specific deficiency of *MGAT1* in mice results in spontaneous neuronal apoptosis *in vivo* and neurological deficits (14). Combined, these data suggest that multiple genetic and environmental factors converge to disrupt *N*-glycan branching, thereby promoting both autoimmune demyelination and neurodegeneration in MS. Therapeutic manipulation of *N*-glycan branching may provide a simple strategy to correct this underlying molecular defect.

Branching by the Golgi enzymes *Mgat1*, 2, 4, and 5 allows increased production of *N*-acetylglucosamine and poly-*N*-acetylglucosamine, ligands for the galectin family of carbohydrate binding proteins (Fig. 1) (15, 16). Galectins bind *N*-glycans in proportion to *N*-acetylglucosamine content, which is enhanced by branching and extension with poly-*N*-acetylglucosamine. Multivalent binding of galectins to *N*-glycans attached to surface glycoproteins forms a molecular lattice at the cell surface that regulates the clustering and endocytosis of transmembrane receptors and transporters to control cell growth and differentiation (12, 17–22). In T cells, *N*-glycan branching inhibits basal and activation signaling through the T cell receptor (TCR) and CD45, promotes growth arrest by cytotoxic T lymphocyte antigen-4 (CTLA-4), and enhances T-helper 2 (Th2) over T-helper 1 (Th1) differentiation (12, 21, 23–28). Metabolically increasing availability of substrate (*i.e.* UDP-GlcNAc) to Golgi enzymes by supplementing cells *in vitro* with the simple sugar *N*-acetylglucosamine (GlcNAc) enhances *N*-glycan branching, suppresses T cell growth, promotes CTLA-4 surface expression, and inhibits adoptive transfer EAE (29). GlcNAc also appears to be active when given orally to mice, with FACS analysis using the plant lectin L-PHA indicating enhanced *N*-glycan branching (29). Indeed, oral GlcNAc suppresses spontaneous autoimmune diabetes in nonobese diabetic mice when initiated prior to disease onset (29). Here, we report that oral GlcNAc has immunomodulatory effects and inhibits EAE when initiated after disease onset, and we confirm increases in *N*-glycan branching by MALDI-TOF mass spectroscopy and anion exchange chromatography. As GlcNAc is a dietary supplement available “over the counter” in the United States and has been used safely in humans orally (30), our data suggest that oral GlcNAc may provide a simple

and inexpensive therapeutic strategy to target an underlying molecular and genetic defect promoting disease.

EXPERIMENTAL PROCEDURES

MALDI-TOF Mass Spectroscopy and High pH Anion Exchange Chromatography (HPAEC)—MALDI-TOF and HPAEC-FL profiling of *N*-glycans were performed by the Glycotechnology Core Resource at the Glycobiology Research and Training Center at the University of California, San Diego. CD3⁺ T cells were treated with 20 mM HEPES buffer at pH 8.2 containing 1% SDS at 100 °C for 5 min. The sample was cooled to room temperature, and SDS was blocked with 1.25% Nonidet P-40 at room temperature for 30 min followed by treatment with peptide *N*-glycosidase F to release the *N*-glycans. Released *N*-glycans were further purified by SepPak C18 cartridge and Poly-Graphitized-Charcoal cartridge. Purified *N*-glycans were characterized by MALDI-TOF mass spectrometry and HPAEC-FL methods. For MALDI mass spectrometry the sample was mixed in a 1:1 ratio with super-DHB matrix and spotted on MALDI plates, and spectra were acquired in the positive mode. Identification of fluorescent labeled *N*-glycans was also done by HPAEC coupled with an online fluorescent detector (HPAEC-FL). A fluorescence dye 2-amino benzamide (2-AB) was tagged to the reducing end of the *N*-glycans followed by purification of 2-amino benzamide-labeled glycans by Glyko clean S-cartridge. The labeled *N*-glycans were dissolved in water and separated using a PA-1 column (4 × 250 mm; Dionex). The eluted peaks were identified by matching with retention times of 2-amino benzamide-labeled *N*-glycans isolated from RNase B (for high mannose glycans) and fetuin (sialylated glycans). CD3⁺ T cells were harvested from age- and sex-matched littermate wild-type PL/J mice orally treated with GlcNAc (Ultimate Glucosamine, Wellesley Therapeutics) for 7 days by supplementing the drinking water at 0.25 mg/ml. 6 mice were used per group, and the harvested CD3⁺ T cells were pooled prior to analyses. To measure serum GlcNAc concentrations, blood was taken from age- and sex-matched littermate wild-type C57BL/6 control mice or mice orally treated with GlcNAc for 7 days by supplementing the drinking water at 0.25 mg/ml. The samples were spun with a 3K spin filter to remove serum proteins and then analyzed by HPAEC. 4 mice were used for the GlcNAc treatment group and 2 mice for the control group. All procedures and protocols with mice were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

EAE Induction and Oral GlcNAc Treatment—EAE was induced by subcutaneous immunization of C57BL/6 wild-type and 2D2 TCR transgenic mice with 100 μg of MOG 35–55 peptide (AnaSpec) emulsified in Complete Freund's adjuvant (Sigma) containing 4 mg/ml heat-inactivated *Mycobacterium tuberculosis* (H37RA; Difco) distributed over two spots on the hind flank. All mice received 150 ng of pertussis toxin (List Biological Laboratories) by intraperitoneal injection on days 0 and 2 after immunization. Mice were examined daily for clinical signs of EAE over the next 30–40 days with the observer blinded to treatment conditions. Mice were scored daily in a blinded fashion as follows: 0, no disease; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness

or paralysis and hindlimb paralysis; 5, moribund or dead. Mice were treated orally with GlcNAc by supplementing the drinking water at 0.25 mg/ml starting on the second day of clinical disease and continued for the duration of the study. Treatment was administered every other day, and oral consumption by all mice was verified by measuring the amount of drinking water left over after each treatment. On average, each mouse drank ~4.5–5 ml in volume. The 2D2 TCR transgenic mice have specificity for MOG 35–55 peptide and develop EAE when immunized with the complete immunization protocol of encephalitogenic peptide plus pertussis toxin. This immunization regimen has been reported previously to result in 90% disease

Antigen-specific Cell Assays—Splenocytes were isolated from GlcNAc or control-treated mice and cultured *in vitro* with MOG 35-55 peptide titrated from 0 to 40 $\mu\text{g/ml}$. Cells were cultured in 24-well plates at a concentration of 3×10^6 cells ml^{-1} for 72–120 h. Culture medium consisted of RPMI

1640 medium supplemented with 10% FBS, 2 μM L-glutamine, 100 units ml^{-1} penicillin/streptomycin, and 2 μM 2-mercaptoethanol.

Flow Cytometry and L-PHA Staining—Cells were washed with FACS buffer (PBS containing 0.1% (w/v) sodium azide and 2% BSA) and stained with anti-CD4 (RM4-5), anti-CD25 (PC61.5), anti-FoxP3 (FJK-16s) from eBioscience and *Phaseolus vulgaris* leucoagglutinating lectin (L-PHA, 4 $\mu\text{g}/\text{ml}$) from Sigma for 30 min on ice. After incubation, cells were washed twice with FACS buffer and analyzed by FACS.

Cytokine Analysis—Supernatants from splenocyte cultures from immunized, and EAE mice were used for cytokine analysis. Cytokine levels were determined by using FlowCytomix Multiplex kit (eBioscience) according to the manufacturer's protocol. Results are shown as mean of triplicate or greater values \pm S.E.

RESULTS

MALDI-TOF Mass Spectrometry and HPAEC Analysis of N-Glycan Structures—To evaluate the effect of oral GlcNAc on N-glycan branching, GlcNAc was provided to age- and sex-matched littermate mice via their drinking water for 7 days. A concentration of 0.25 mg/ml was used (~ 50 mg/kg/day), which has previously been observed to maximally enhance binding of L-PHA (*P. vulgaris*, leucoagglutinin) to *ex vivo* T cells. L-PHA is a plant lectin that binds specifically to $\beta 1,6$ -GlcNAc-branched N-glycans produced by Mgat5 and serves as a marker of N-glycan branching (12, 32, 33). HPAEC estimated the serum GlcNAc concentration to be 0.66 ± 0.20 mM with GlcNAc treatment ($n = 4$; supplemental Fig. 1). N-Glycans were isolated from purified CD3⁺ T cells and analyzed by MALDI-TOF mass spectrometry in positive and negative mode, comparing control and GlcNAc-treated cells (Fig. 2, A–D). N-Glycan structures predicted from the mass are shown in the profiles and reveal predominantly high mannose type carbohydrates. These N-glycans are near the beginning of the N-glycan biosynthetic pathway, prior to modification by Mgat1 and downstream branching enzymes. However, a major molecular ion signal at m/z 1809.86 in positive mode was detected in the GlcNAc-treated sample that was absent in the control sample, a mass consistent with a fucosylated biantennary branched N-glycan. At higher masses in negative mode, GlcNAc therapy also increased the level of an unusual signal at 2423 m/z (Na^+ adduct), a mass consistent with a monoantennary branched structure containing poly-N-acetylactosamine and a single sialic acid (Fig. 2, C and D). The presence of sialic acid rules out an alternative biantennary N-glycan terminated by two $\alpha 1,3$ -galactoses, a structure previously described in T cell blasts (34).

The increase in branched N-glycans was further supported by HPAEC analysis of fluorescently tagged N-glycans from the two treatment groups (Fig. 2, E and F). High mannose and sialylated branched N-glycans were assigned based on N-glycan standards from RNase B, IgG, and fetuin and correlated with the structures observed by mass spectroscopy. The GlcNAc-treated samples had higher amounts of sialylated branched N-glycans relative to high mannose oligosaccharides. Peak 9 eluted at the same time as monosialylated branched N-glycans from fetuin and IgG, suggesting that this corre-

sponds to the major 2423 m/z structure in the MALDI-TOF spectrum. These standards also suggest that peak 8 represents the nonsialylated biantennary signal observed at 1810 m/z (Fig. 2, A and B). Although mass spectroscopy and linkage analysis of individual peaks are required to assign structures to these peaks definitively, the combined data support the conclusion that oral GlcNAc enhanced N-glycan branching and extension with poly-N-acetylactosamine in T cells *in vivo*. Indeed, these data are consistent with previously published mass spectrometry data of *in vitro* GlcNAc supplementation revealing increased branched N-glycans in tumor cells and UDP-HexNAc substrate levels in activated mouse T cells (21, 29).

Oral GlcNAc Begun at Initiation of EAE Inhibits Th1/Th17 Responses—The uptake of GlcNAc into cells is by macropinocytosis, a process that depends on the rate of membrane turnover. Although resting T cells have minimal membrane turnover, rapidly dividing T cell blasts have high membrane turnover; suggesting that GlcNAc may more effectively enhance N-glycan branching in the latter. Indeed, *in vitro* analysis reveals that GlcNAc increased L-PHA binding in T cell blasts at 4-fold lower concentrations than resting T cells (Fig. 3A).

The above data suggest that for treatment of EAE and MS, myelin-activated T cell blasts (with increased macropinocytosis) should be more responsive to GlcNAc than resting T cells, thereby providing a degree of specificity for disease causal cells. Therefore, we examined the role of *in vivo* GlcNAc treatment in the regulation of T cells during the initiation phase of EAE in C57BL/6 mice, a mouse model induced by MOG 35-55 with adjuvant to mimic CNS pathology in MS. Oral GlcNAc treatment was initiated at the same time as immunization to determine early effects on T cells. After 14 days, splenocytes harvested from mice treated with GlcNAc had reduced CD25⁺ T cells upon restimulation with antigen (Fig. 3, B and C) and promoted development of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Fig. 3D). Oral GlcNAc treatment *in vivo* also inhibited myelin-antigen induced secretion of Th1 (IFN- γ and TNF- α) and Th17 (IL-17 and IL-22) cytokines that promote disease (Fig. 3E).

Oral GlcNAc Suppresses Th1/Th17 and Treats EAE When Initiated after Disease Onset—To examine the effect of oral GlcNAc when initiated after development of clinical MOG 35-55-induced EAE, 0.25 mg/ml GlcNAc was provided in the drinking water starting on the second day of clinical disease and continued for the duration of the study. This therapeutic approach significantly reduced clinical symptoms and increased N-glycan branching in T cells as seen by L-PHA staining (Fig. 4, A and B). Consistent with this, a significant reduction in CD25⁺ T cells was observed in representative GlcNAc-treated mice taken at the peak of disease compared with control mice (Fig. 4C). Compared with control mice, *in vivo* treatment with oral GlcNAc after disease onset also suppressed proinflammatory recall responses to the encephalitogenic MOG 35-55 peptide, with significant reductions in secretion of IFN- γ , TNF- α , IL-17, and IL-22 (Fig. 4D).

To examine whether oral GlcNAc is effective in more severe forms of disease, the 2D2 TCR transgenic EAE model was utilized (31). Immunization of these mice with MOG 35-55 results

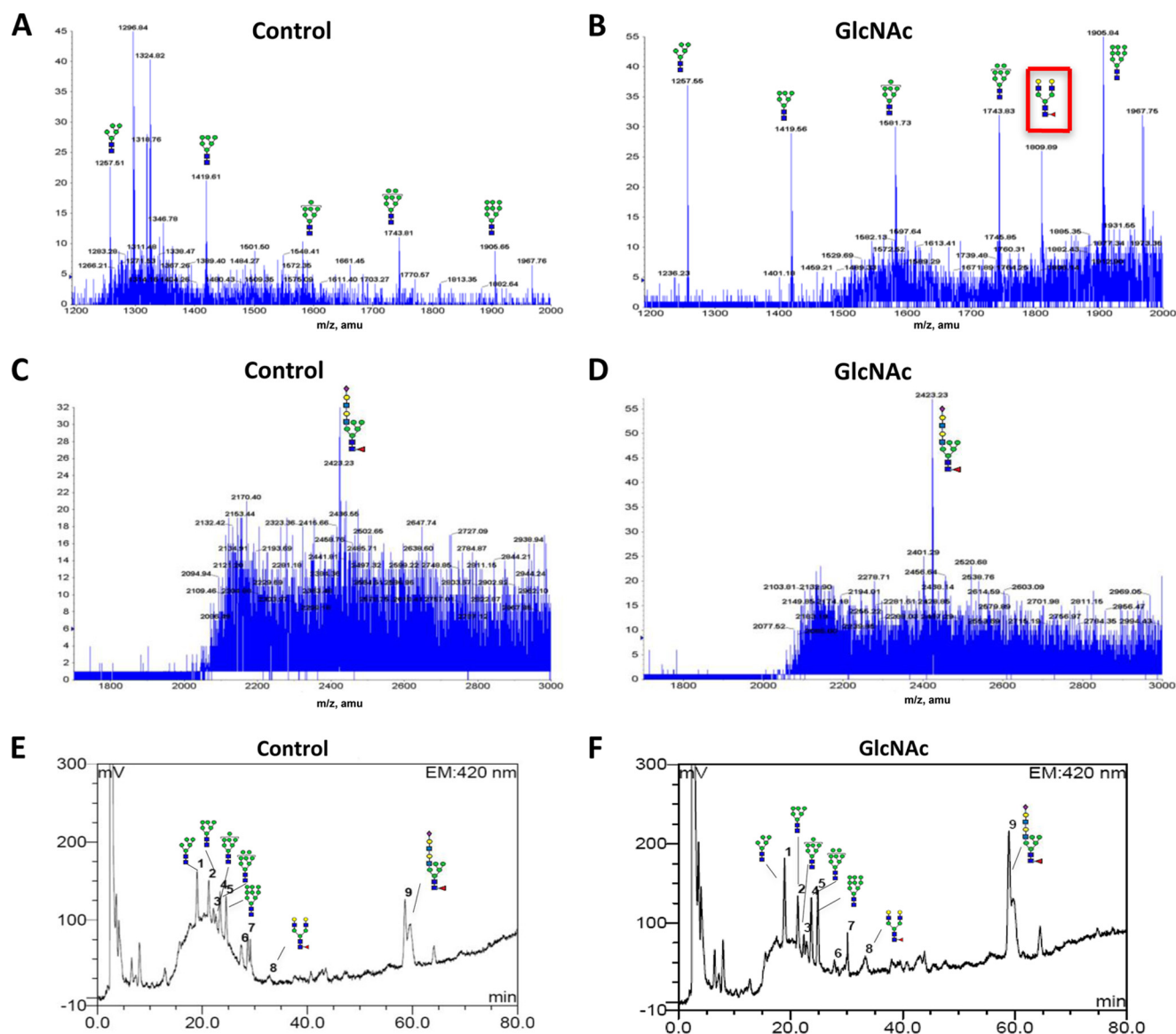


FIGURE 2. MALDI-TOF mass spectrum and HPAEC profile of *N*-glycans. *N*-Glycans were isolated from purified CD3⁺ T cells from age- and sex-matched wild-type control mice or mice treated orally with GlcNAc for 7 days and analyzed by MALDI-TOF mass spectrometry (A–D) or HPAEC (E and F). Oral GlcNAc was administered by supplementing the drinking water at 0.25 mg/ml, with intake confirmed by measuring the amount of drinking water consumed. 6 mice were used per group, and the harvested CD3⁺ T cells were pooled prior to analyses. The assignment of likely structures in the MALDI-TOF spectrum is based on mass and those in HPAEC on *N*-glycan standards from RNase B, IgG, and fetuin and correlations with the major structures observed in the MALDI-TOF spectrum. Definitive structure assignment of the latter requires HPAEC-mass spectrometry in-line as well as linkage analysis. Abbreviations for individual sugars are defined in Fig. 1.

in severe and often fatal EAE. Initiating oral GlcNAc after clinical onset of MOG 35-55-induced EAE in 2D2 TCR transgenic mice inhibited clinical progression in mice with nonlethal disease but had little effect in mice with rapid progression to death (Fig. 5, A and B). As GlcNAc supplementation for at least ~5–7 days is required to enhance *N*-glycan branching in T cells (29), the inability to inhibit rapidly progressing disease may result from the delay in onset of action. To confirm enhanced expression of *N*-glycan branching in the surviving EAE mice treated with oral GlcNAc, two representative mice each from treatment and control groups were taken at the end of the study. A ~24% increase in L-PHA binding (*i.e.* Mgat5-branched *N*-glycans) in CD4⁺ T cells were observed with oral GlcNAc treatment (Fig. 5C).

DISCUSSION

Genetic data in mice and humans provides strong evidence for a role of *N*-glycan branching in demyelinating disease pathogenesis and disease progression (11–13,17–22). Here, we provide data indicating that oral administration of the simple sugar GlcNAc enhances *N*-glycan branching while inhibiting CD25⁺ cells, Th1 and Th17 cytokines, and disease progression in EAE when initiated after disease onset. This result is consistent with earlier *in vitro* GlcNAc supplementation data demonstrating suppression of TCR signaling, T cell proliferation, CTLA-4 endocytosis, Th1 differentiation, and adoptive transfer EAE (21, 29). A pilot study of oral GlcNAc in pediatric treatment-resistant inflammatory bowel disease further reveals

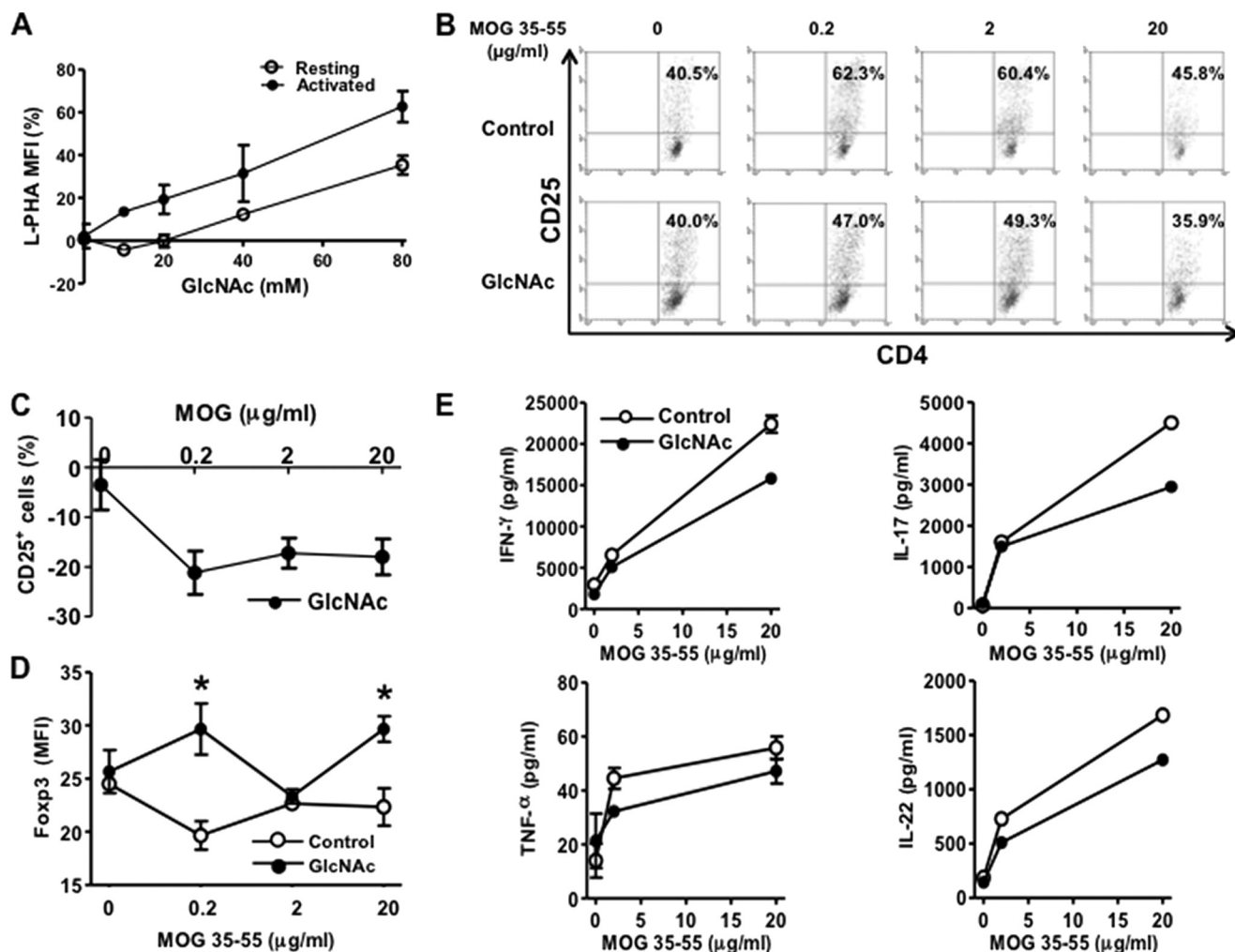


FIGURE 3. Oral GlcNAc treatment inhibits CD25 expression and proinflammatory cytokine production. A, splenocytes were isolated from mice and cultured *in vitro* with the indicated concentrations of GlcNAc under resting or activated (1000 ng/ml anti-CD3) conditions for 3 days, stained with L-PHA-FITC in triplicate, and analyzed by FACS. B–D, mice were immunized with MOG 35–55 peptide emulsified in Complete Freund's adjuvant and treated orally with GlcNAc at the same time by supplementing the drinking water at 0.25 mg/ml. After 14 days, splenocytes harvested from mice treated with GlcNAc had reduced CD25⁺ T cells upon restimulation with MOG 35–55 peptide in culture (B and C) and promoted development of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (D). The reduction in CD25⁺ T cell blasts at the highest antigen stimulation levels is likely secondary to the observed increase in cell death, consistent with antigen-induced cell death at high antigen stimulation levels. E, oral GlcNAc treatment *in vivo* also inhibited secretion of Th1 cytokines (IFN-γ and TNF-α) and Th17 cytokines (IL-17 and IL-22) upon restimulation with MOG 35–55 peptide *in vitro*. 3 mice were used per group, and the harvested CD3⁺ T cells were pooled within each group prior to analyses. *p* values in C were determined by *t* test. *, *p* < 0.01. Error bars represent the means ± S.E. of duplicate or greater values unless otherwise stated.

the potential of GlcNAc as a therapeutic agent in humans (35). Remarkably, this study reported that 8 of 12 children with severe inflammatory bowel disease went into clinical remission with evidence of histological improvement. Indeed, 3 of the responders relapsed within ~1 month following disruption of GlcNAc therapy, but improved again once GlcNAc therapy was reinitiated. Together, these data provide compelling evidence for GlcNAc as a potential simple and cost-effective oral therapy for MS and the need for a large clinical trial to test this hypothesis. If proven effective, it would provide the first therapy that directly targets an underlying genetic mechanism promoting disease.

The molecular mechanism underlying the therapeutic effect of GlcNAc has previously been investigated (20, 21, 29). GlcNAc-mediated inhibition of TCR signaling, T cell proliferation, Th1 differentiation, and CTLA-4 endocytosis are reversed by Golgi inhibitors that block *N*-glycan branching (*i.e.*

swainsonine, deoxymannojirimycin) (29), confirming that GlcNAc alters T cell function by enhancing *N*-glycan branching.

Like GlcNAc, glucosamine (GlcN) may also supplement the hexosamine pathway to increase UDP-GlcNAc and *N*-glycan branching while inhibiting autoimmunity (29, 36, 37). GlcNAc enters the cell through macropinocytosis and is salvaged into the hexosamine pathway after phosphorylation by *N*-acetylglucosamine kinase to GlcNAc 6-phosphate (Fig. 1). GlcN enters the cell through glucose transporters, allowing an ~200–400-fold reduction in effective concentration relative to GlcNAc. However, GlcN 6-phosphate also may flux into the glycolytic pathway for ATP production following deamination by glucosamine-6-phosphate deaminase (Fig. 1) (38, 39). In contrast, GlcNAc does not enter glycolysis, the pentose phosphate pathway or the TCA cycle, and is exclusively salvaged into the hexosamine pathway for production of UDP-GlcNAc (40).

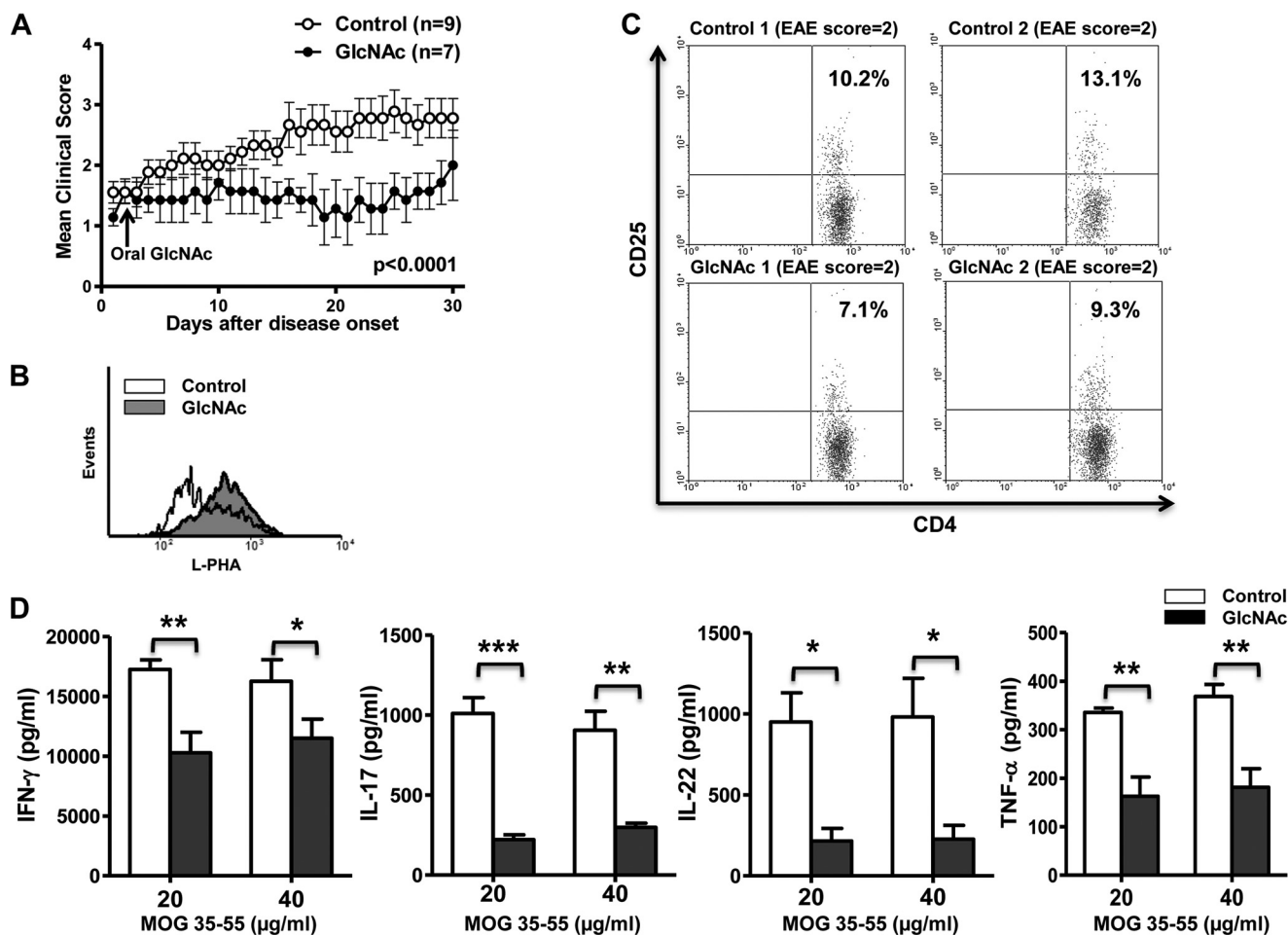


FIGURE 4. Oral GlcNAc treatment attenuates the clinical course of EAE. A, EAE was induced in C57BL/6 mice by immunization with MOG 35-55 peptide emulsified in Complete Freund's adjuvant and pertussis toxin. Mice were treated orally with GlcNAc by supplementing the drinking water at 0.25 mg/ml starting on the second day after disease onset and continued for the duration of the study ($n = 9$ per control group, $n = 7$ per GlcNAc group). Day 1 indicates the first day of disease onset. Mice were examined daily for clinical signs of EAE over the next 30 days with the observer blinded to treatment conditions and scored daily as follows: 0, no disease; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness or paralysis and hindlimb paralysis; 5, moribund or dead. Mean clinical scores per group daily were compared by the Mann-Whitney U test. B, GlcNAc treatment increased N -glycan branching in T cells as seen by L-PHA staining in representative mice taken at the peak of disease. The results are representative of at least three mice compared from each group. C, a significant reduction in CD25⁺ T cells was observed in representative GlcNAc-treated mice taken at the peak of disease compared with control mice. The results are representative of at least three mice compared from each group. D, *in vivo* treatment with GlcNAc inhibited production of proinflammatory cytokines IFN- γ , TNF- α , IL-17, and IL-22 upon restimulation with MOG 35-55 peptide *in vitro*. p values in D were determined by t test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Error bars represent the means \pm S.E. of duplicate or greater values unless otherwise stated.

Autophagy and endoplasmic reticulum-associated degradation may further recycle GlcNAc within cells. In this regard, the effectiveness of GlcN in increasing N -glycan branching is limited compared with GlcNAc. Indeed, our previous *in vitro* studies revealed that increasing concentrations of GlcN initially increased but then reduced N -glycan branching (29). The latter likely arises from GlcN 6-phosphate acting as a potent inhibitor of glutamine fructose 6-phosphate amidotransferase, the rate-limiting enzyme shunting fructose 6-phosphate into the hexosamine pathway (41). In contrast to GlcN, increasing GlcNAc concentrations *in vitro* is observed to only enhance N -glycan branching.

The estimated *in vivo* serum concentrations of GlcNAc achieved in our experiments (0.66 ± 0.20 mM) appear to be significantly less than that required *in vitro* to enhance N -glycan branching. Consistent with our *in vivo* data, Shoji *et al.* have reported $\sim 25\%$ tissue incorporation/ $\sim 75\%$ elimination of oral N -[^{14}C]acetylglucosamine (42). The *in vivo* concentrations of

GlcNAc during EAE are likely to be less than those required *in vitro* for several reasons. First, uptake of GlcNAc via macropinocytosis is enhanced in T cell blasts, which allows myelin antigen-activated T cells to be more sensitive than other cells during EAE. Second, time of exposure to GlcNAc enhances uptake by macropinocytosis. *In vitro* exposure of GlcNAc is limited to 3–5 days due to increasing T cell death in culture, an issue avoided *in vivo*. Thus, longer term GlcNAc exposure is only possible *in vivo*, further lowering the concentration required to enhance N -glycan branching. Consistent with this, N -glycan branching in resting T cells does not increase until after ~ 7 days of therapy. Finally, the mg/kg doses of GlcNAc that achieve maximal increases in N -glycan branching *in vivo* also suppress autoimmunity in mice and inflammatory bowel disease in humans (29, 35). This provides a strong correlation between oral GlcNAc supplementation, increases in N -glycan branching *in vivo* and suppression of EAE and autoimmunity; results supported by genetic data demonstrating that N -glycan

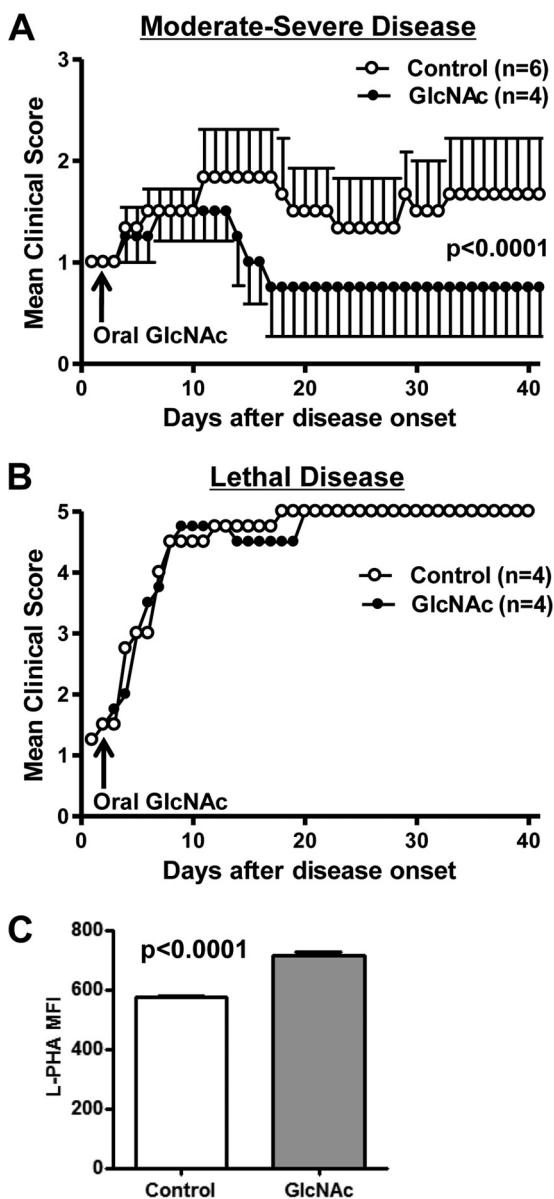


FIGURE 5. Oral GlcNAc treatment after disease onset attenuates the clinical course of EAE in moderate-severe disease, but not disease with rapid lethal progression. A and B, EAE was induced in 2D2 TCR transgenic mice by immunization with MOG 35-55 peptide emulsified in Complete Freund's adjuvant and pertussis toxin. Mice were treated orally with GlcNAc by supplementing the drinking water at 0.25 mg/ml starting on the second day after disease onset and continued for the duration of the study ($n = 10$ per control group, $n = 8$ per GlcNAc group). Day 1 indicates the first day of disease onset. Mice were examined daily for clinical signs of EAE over the next 40 days with the observer blinded to treatment conditions and scored daily as follows: 0, no disease; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness or paralysis and hindlimb paralysis; 5, moribund or dead. Mice that scored ≤ 4 were included in the moderate-severe disease group (A), and mice with scores of 5 (i.e. died within the first 40 days after disease onset) (B) were included in the lethal disease group. Mean clinical scores per group daily (A and B) were compared by the Mann-Whitney U test. C, 2 representative mice from each group of the 2D2 TCR transgenic EAE experiment were used to assess expression of branched N -glycans. Splenocytes were harvested from the immunized mice and stained with anti-CD4 and L-PHA lectin in triplicate and analyzed by FACS. p values in C were determined by t test. Error bars represent the means \pm S.E. of triplicate or greater values unless otherwise stated.

branching suppresses T cell function and autoimmunity. However, our data does not exclude additional potential mechanisms for GlcNAc-mediated suppression of autoimmunity.

GlcNAc is readily available over the counter in North America and has been used orally as a dietary supplement for many years without reported adverse effects. Large dose intravenous GlcNAc administration demonstrated no toxicity issues and no alterations in blood glucose concentrations or insulin resistance (30). Chronic systematic toxicological studies up to 104 weeks in animals have further validated GlcNAc as a safe compound with no associated toxicity, including lack of increased cancer risk (43, 44). As polymerized GlcNAc forms chitin present in insects and crustaceans, GlcNAc is highly abundant. GlcNAc can be used alone or in addition to other immunoregulatory drugs to increase efficacy and decrease dosage and toxicity. As GlcNAc may provide the first therapeutic approach that directly targets an underlying disease causal mechanism, human clinical trials are warranted.

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